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Regulation of plant immunity via small RNA-mediated control of NLR expression

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Abstract

Plants use different receptors to detect potential pathogens: membrane-anchored pattern recognition receptors (PRRs) activated upon perception of pathogen-associated molecular patterns (PAMPs) that elicit pattern-triggered immunity (PTI); and intracellular nucleotide-binding leucine-rich repeat proteins (NLRs) activated by detection of pathogen-derived effectors, activating effector-triggered immunity (ETI). The interconnections between PTI and ETI responses have been increasingly reported. Elevated NLR levels may cause autoimmunity, with symptoms ranging from fitness cost to developmental arrest, sometimes combined with run-away cell death, making accurate control of NLR dosage key for plant survival. Small RNA-mediated gene regulation has emerged as a major mechanism of control of NLR dosage. Twenty-two nucleotide miRNAs with the unique ability to trigger secondary siRNA production from target transcripts are particularly prevalent in NLR regulation. They enhance repression of the primary NLR target, but also bring about repression of NLRs only complementary to secondary siRNAs. We summarize current knowledge on miRNAs and siRNAs in the regulation of NLR expression with an emphasis on 22 nt miRNAs and propose that miRNA and siRNA regulation of NLR levels provides additional links between PTI and NLR defense pathways to increase plant responsiveness against a broad spectrum of pathogens and control an efficient deployment of defenses.

Keywords: Effector-triggered immunity, miRNA, NLR proteins, plant immunity, post-transcriptional gene silencing, R genes, RNAi, secondary siRNA.

Introduction

Plants use cell surface and intracellular receptors to detect potential pathogens. Activation of membrane-anchored pattern recognition receptors (PRRs) is typically associated with perception of pathogen-associated molecular patterns (PAMPs) and elicits pattern-triggered immunity (PTI). Intracellular nucleotide-binding (NB) leucine-rich repeat (LRR) receptors (NLRs) detect pathogen-derived effectors, thereby activating effector-triggered immunity (ETI). Pathogen effectors act intracellularly to manipulate host processes and facilitate pathogen spread. The immune response triggered by the activation of NLRs, known as ETI, is fast and robust, and is often accompanied by activation of...
localized programmed cell death known as the hypersensitive response (HR) (Dodds and Rathjen, 2010; Cui et al., 2015). Molecularly, NLRs display a multidomain structure that includes a conserved NB and an LRR domain accompanied in most cases by N-terminal domains that fall into one of three major classes (Meyers et al., 2003; Cui et al., 2015; Zhang et al., 2017): Toll/interleukin-1 (TIR) domains (TNLs), coiled-coil (CC) domains (CNLs), and RPW8-like CC-type domains (RPW8-NLRs or RNLs) (Fig. 1) (Shao et al., 2016; Jubic et al., 2019).

Genes encoding NLRs or, in rare cases, TIR-only or TIR-NB variants (Cesari, 2018), make up most of the Resistance (R) genes that mediate gene-for-gene resistance to pathogens carrying cognate effectors, in this case known as avirulence factors (Flor, 1971). NLRs may act as both sensors and signal transducers. Sometimes they act in pairs such that a sensor NLR is coupled to a so-called helper NLR that triggers signaling. Remarkably, such coupled sensor–helper NLRs are often encoded by tightly linked genes. The principle of coupled sensor and helper NLRs may be extended to become networks, where a large number of sensor NLRs require multiple helper NLRs, not always encoded within clusters in the genome, to mediate resistance against an assortment of effectors and pathogens (Adachi et al., 2019). This increased complexity may reflect NLR evolution driven by the need to keep up with rapidly evolving pathogens (Adachi et al., 2019).

Plant NLRs remain in an inactive form through intramolecular associations prior to effector recognition. Effector recognition by plant NLRs may take place through direct interaction with the effector, by recognition of effector-mediated modifications of a host target protein (guard model) or a protein that mimics its target protein (decoy model), or through direct modification by the effector of a decoy

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**Fig. 1.** NLRs are targeted by miRNAs at highly conserved motifs. According to its N-terminal domain, the three main clades of plant NLRs are depicted. Conserved motifs and experimentally validated miRNAs are highlighted. TNL, Toll/interleukin-1 (TIR) type NLR; CNL, coiled-coil (CC) type NLR; RNL, RPW8-like CC-type NLR. The figure was created with BioRender.com.
domain integrated within the NLR protein (Cesari, 2018). Activation upon recognition proceeds in a complicated process that involves nucleotide exchange, ATP binding, and chaperone-assisted conformational changes, ultimately resulting in multimerization (Ve et al., 2015; Bentham et al., 2018).

Recent structural and molecular studies on CNLs and TNLs have provided detailed insight into NLR multimerization and function. Upon effector activation of the CNL ZAR1, inactive monomers form a pentameric wheel-like complex termed a resistosome (Wang et al., 2019a, b). This pentamerization triggers the release of the terminal α-helices of the CC domain, which go from being buried within the inactive monomer to projecting out of the resistosome plane to form a funnel-like structure required for cell death-inducing activity (Wang et al., 2019a, b). The funnel inserts itself into the plasma membrane forming a pore or channel, potentially releasing host defense-potentiating molecules and/or promoting inward Ca\(^{2+}\) fluxes to drive intracellular signaling cascades (Wang et al., 2019a, b; Bi et al., 2021; Dongus and Parker, 2021). Key advances have also been made on plant TIR resistosomes: upon effector-induced TNL tetramerization (Ma et al., 2020; Martin et al., 2020), a holoenzyme is formed in which the TIR domains have an enzymatic activity that cleaves NAD\(^{+}\) into nicotinamide (Nam) (Essuman et al., 2018) and a variety of ADP-ribose (ADPR) isomers (Manik et al., 2022), and related molecules (Horsefield et al., 2019; Wan et al., 2019; Huang et al., 2022; Jia et al., 2022; Leavitt et al., 2022), with signaling functions. Remarkably, TIRs may also polymerize to form fibers that have the distinct enzymatic activity of catalyzing hydrolysis of dsRNA or, in vitro, DNA, to form 2',3'-cAMP or 2',3'-cGMP (Essuman et al., 2022; Yu et al., 2022) with cell death-inducing function. Some of the molecules produced by TIR enzymatic activities are perceived by EDS1-PAD4 or EDS1-SAG101 heterodimeric nucleotide receptors that bind to and activate distinct helper NLRs upon nucleotide binding (Huang et al., 2022; Jia et al., 2022, 2023), while the biochemical function of others remains to be determined.

Activation of NLR-mediated pathways is under tight control. Many NLRs are involved in trade-offs between disease resistance and growth or response to abiotic stresses (Ariga et al., 2017; Karasov et al., 2017), and autoimmunity as a consequence of constitutive or misregulated activation can have deleterious effects, including severe growth suppression (Shirano et al., 2002; Tian et al., 2003; Korves and Bergelson, 2004; Palma et al., 2010). NLR protein production undergoes regulation at the transcriptional, post-transcriptional, translational, and post-translational levels (Cheng et al., 2011; Gloggnitzer et al., 2014; Wu et al., 2017; Lai and Eulgem, 2018). Among these mechanisms, small RNA-guided repression at the post-transcriptional level has been found to be implemented across many different plant species to achieve appropriate NLR expression and regulation (Zhai et al., 2011; Li et al., 2012b; Shivaprasad et al., 2012; Fei et al., 2013). Small RNAs are at the core of RNAi phenomena that are of significant interest in plant–pathogen interactions because of the employment of effectors targeting RNAi pathways in many unrelated groups of pathogens, including viruses, bacteria, fungi, and oomycetes (Anandalakshmi et al., 1998; Voinnet et al., 1999; Navarro et al., 2008; Qiao et al., 2013; Csorba et al., 2015; Yin et al., 2019). Two types of small RNAs, miRNAs and siRNAs, are involved in the post-transcriptional regulation of NLRs (Zhai et al., 2011; Li et al., 2012b; Shivaprasad et al., 2012; Fei et al., 2013), and the mechanistic underpinnings of this type of regulation and its possible significance in the broader perspective of plant–pathogen interactions will be the focus of the remainder of this review.

**Elements of the biogenesis and action of miRNAs**

Small silencing RNAs are ~21–24 nt single-stranded non-coding RNAs that explain the sequence specificity of RNAi by their base pairing to complementary RNA targets. These small RNAs always act in association with proteins of the ARGONAUTE (AGO) family (Vaucheret, 2008; Poulsen et al., 2013; Martin-Merchan et al., 2023). RNAi phenomena include both transcriptional and post-transcriptional repression, and, in plants, different classes of small RNAs can be defined according to their biogenesis and/or mode of action (Axtell, 2013; Bologna and Voinnet, 2014; Borges and Martienssen, 2015). miRNAs are 20–24 nt RNAs mainly involved in post-transcriptional regulation. They are key regulators of important plant processes, including plant development and stress responses (Voinnet, 2009). miRN4 loci are transcribed by RNA polymerase II, yielding poly(A)-tailed and m\(^{G}\)-capped primary miRNA transcripts (pri-miRNAs) containing a hairpin-like structure (Voinnet, 2009). Pri-miRNAs are processed into small RNA duplexes of ~21 nt by the dsRNA-directed RNAase DICER-LIKE1 (DCL1) (Park et al., 2002; Reinhart et al., 2002). In turn, the duplex consisting of miRNA and miRNA* strands undergoes protective ribose methylation at both 3’ ends, catalyzed by the methyl transferase HUA ENHANCER1 (HEN1) (Yu et al., 2005). The resulting methylated duplex is then loaded onto AGO1, the main ARGONAUTE protein operating in the miRNA pathway in plants (Vaucheret et al., 2004; Baumberger and Baulcombe, 2005). Subsequently, the miRNA* strand is ejected from AGO1, thus producing a mature miRNA-induced silencing complex (miRISC). The mature miRISC is the regulatory machine that exerts repression of target RNAs by endonucleolytic cleavage (slicing) or translational repression (Fig. 2) (Chen 2004; Vaucheret et al., 2004; Baumberger and Baulcombe, 2005; Qi et al., 2005; Brodersen et al., 2008).
The special 22 nt miRNAs and triggering of the RNA-dependent RNA polymerase amplification module

Some plant pri-miRNAs contain asymmetric bulges. This may lead to the production of 22 nt miRNA/21 nt miRNA* duplexes such that an miRISC containing a 22 nt miRNA is formed. While this single nucleotide difference in small RNA size may seem minor, it is anything but that, since 22 nt miRNAs have a unique and powerful ability: triggering the conversion of their RNA targets into dsRNA by recruiting RNA-dependent RNA polymerase 6 (RDR6), in a process assisted by the proteins suppressing gene silencing 3 (SGS3) and silencing defective 5 (RDR6), in a process assisted by the proteins suppressing gene silencing 3 (SGS3) and silencing defective 5 (RDR6). This dsRNA is cut into 21 nt sRNA duplexes by DCL2 that gives rise to small RNA duplexes 22 nt in size (Fig. 2) (Zhai et al., 2013; Tu et al., 2015; Fei et al., 2018; Wang et al., 2018). Nonetheless, an asymmetric duplex structure may confer additional properties on the resulting miRISC that enhance its ability to induce secondary siRNAs (Manavella et al., 2012).

PhasiRNA production may have at least three distinct outcomes. First, the silencing of the primary target RNA is amplified, a cis effect that is quantitative in nature. Second, additional target RNAs unrelated in sequence to the primary 22 nt trigger miRNA may be silenced by the phasiRNAs, a trans effect that is qualitative in nature (Fig. 2) (Allen et al., 2005; Yoshikawa et al., 2005; Zhai et al., 2011). Third, siRNAs produced via the RDR6-dependent amplification module show a markedly non-cell-autonomous mode of action, thus potentially extending the spatial boundaries of the domain of small RNA-mediated repression (Felippes et al., 2011).

Evolution of miRNA-mediated NLR regulation

Phylogenetic analysis indicates that NLR emergence dates back to the common ancestor of the whole green lineage, followed by a rapid divergence into the TNL, CNL, and RNL subclades (Shao et al., 2019). The evolution of these receptors is highly dynamic and thoroughly influenced (and re-shaped) by pathogen–host interactions on an evolutionary time scale (Jacob et al., 2013; Borrelli et al., 2018; Tamborski and Krasileva, 2020). Accordingly, NLRs are polymorphic at the population level, and they define the repertoire of effectors that a given individual can detect (Kuang et al., 2004; Zhang et al., 2016). As with other gene families, a positive correlation exists between the number of NLR genes and the total number of genes in a plant genome (Wang et al., 2011). Even though NLR genes are the largest gene family giving rise to phasiRNAs (Fei et al., 2013; Liu et al., 2020), miRNA-based NLR regulation can be only traced back to the emergence of gymnosperms (Yue et al., 2012; Xia et al., 2015; Zhang et al., 2016), and phasiRNA biogenesis from NLRs seems to be more prevalent in eudicots than in monocots (Liu et al., 2020). Strikingly, a high degree of variability exists between the numbers of miRNA–NLR–phasiRNA networks within different species, which range from two miRNAs and a few NLRs triggering the production of phasiRNAs in Arabidopsis thaliana, to ~19 miRNAs and >750 NLRs doing so in Picea abies (Xia et al., 2015). The expansion
of plant NLRomes and the high variability of NLR gene repertoires are governed by intricate processes of duplication, recombination, and genomic rearrangements (Friedman and Baker, 2007; Borrelli et al., 2018). These same processes are also believed to underpin the emergence of NLR-targeting miRNAs (e.g. NLRs) are sliced by a transitive inducer miRNA (see A), triggering the conversion of one of the cleavage fragments into a dsRNA by the action of RDR6, in a process assisted by SGS3 and SDE5 (not depicted). The dsRNA becomes a substrate of DCL4 to render secondary phased siRNAs (phasiRNAs) that load onto AGO1/AGO2 to regulate either the same mRNA (cis silencing) or other mRNAs (trans silencing), thus amplifying or reinforcing the silencing signal of the miRNA, respectively. PhasiRNA-dependent regulation may happen by slicing or translational inhibition (unknown). Notice that phasiRNA movement is also depicted (as proposed) in the model; however, whether phasiNLRs possess this property remains unknown. DCL, DICER-LIKE; HEN1, HUA ENHANCER 1; POLII, POLYMERASE II; AGO, ARGONAUTE; SGS3, SUPPRESSOR OF GENE SILENCING 3; RDR6, RNA DEPENDENT RNA POLYMERASE 6; SDE5, SILENCING DEFECTIVE 5. The figure was created with BioRender.com.
Table 1. Validated miRNAs that trigger phasiNLR biogenesis.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Species</th>
<th>miRNA size</th>
<th>Main miRNA targets</th>
<th>Target site domain/motif</th>
<th>Evidence of miRNA activity</th>
<th>PHAS loci</th>
<th>References</th>
</tr>
</thead>
<tbody>
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<td>miR172</td>
<td>S. lycopersicum/G. max</td>
<td>21-nt</td>
<td>TNL</td>
<td>Kinase-2</td>
<td>PARE</td>
<td>Glyma18g14655</td>
<td>Seo et al. (2018)</td>
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<tr>
<td>miR472</td>
<td>A. thaliana/P. trichocarpa</td>
<td>22 nt</td>
<td>CNLs</td>
<td>P-loop</td>
<td>5’-RACE and/or PARE</td>
<td>RPS5, RSG1-2, AT5G34740; PipHa3-6</td>
<td>Lu et al. (2006); Boccara et al. (2014); Shuai et al. (2016); Su et al. (2018)</td>
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<td>P. trichocarpa/S. lycopersicum</td>
<td>22 nt</td>
<td>TNL/CNL</td>
<td>P-loop</td>
<td>5’-RACE and/or PARE</td>
<td>Several in each species</td>
<td>Lu et al. (2005); Shivaprasad et al. (2012); Jiang et al. (2018); Canto-Pastor et al. (2019)</td>
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<td>miR825-5p</td>
<td>A. thaliana</td>
<td>22 nt</td>
<td>TNLs</td>
<td>TIR-2</td>
<td>PARER and reporter</td>
<td>MIST1</td>
<td>Howell et al. (2007); Chen et al. (2010); Niu et al. (2016); Nie et al. (2019); López-Marquez et al., 2021</td>
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<td>miR1507</td>
<td>M. truncatula</td>
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<td>Kinase-2</td>
<td>PARE</td>
<td>Medtr3g018980</td>
<td>Li et al. (2011)</td>
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<td>miR1885</td>
<td>B. rapa</td>
<td>22 nt</td>
<td>TIR/TNL</td>
<td>TIR-1</td>
<td>5’-RACE and reporter</td>
<td>BraTIR1, BraTNL1</td>
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<td>M. truncatula</td>
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<td>TNL</td>
<td>TIR-1</td>
<td>PARE</td>
<td>Medtr1g044860</td>
<td>Zhai et al. (2011)</td>
</tr>
<tr>
<td>miR5300</td>
<td>S. lycopersicum/S. tuberosum</td>
<td>22 nt</td>
<td>CNL</td>
<td>P-loop</td>
<td>PARE</td>
<td>Several in each species</td>
<td>Zhai et al. (2011); Liu et al. (2014); Canto-Pastor et al. (2019)</td>
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<td>miR6019</td>
<td>N. tabacum</td>
<td>22 nt</td>
<td>TNL</td>
<td>TIR</td>
<td>5’-RACE and/or PARE and/or reporter</td>
<td>Tm2</td>
<td>Seo et al. (2018)</td>
</tr>
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<td>miR6024-3p</td>
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<td>CNL</td>
<td>P-loop</td>
<td>5’-RACE</td>
<td>Rx1; Nb5</td>
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<td>S. lycopersicum</td>
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<td>P-loop</td>
<td>PARE</td>
<td>Tm2; R pivnt1</td>
<td>Li et al. (2012b)</td>
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<td>PARE</td>
<td>Sw5</td>
<td>Li et al. (2012b)</td>
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<td>miR9863</td>
<td>H. vulgare</td>
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<td>CNL</td>
<td>ARC</td>
<td>5’-RACE and reporter</td>
<td>MLA</td>
<td>Liu et al. (2014)</td>
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<td>PARE</td>
<td>CA06g00990</td>
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<td>G. max</td>
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<td>PARE</td>
<td>Glyma04g09740</td>
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<td>MA_10435336g0010 (SUMM2)</td>
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<td>NLR</td>
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<td>RNL</td>
<td>LRR</td>
<td>PARE</td>
<td>MA_10429865g0020 (RPP13)</td>
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<td>miR11506</td>
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<td>TNL</td>
<td>TIR</td>
<td>PARE</td>
<td>MA_57122g0010 (TMV resistance N-like)</td>
<td>Xia et al. (2015)</td>
</tr>
</tbody>
</table>
for miRNA–target pairing imposes any constraints in NLR diversification is unclear (Zhang et al., 2016). In any case, targeting of conserved sequences coding for motifs relevant for protein function, as in many miRNA–NLR–mRNA interactions (Table 1), provides an evolutionary link between protein function and miRNA-mediated regulation.

Mode of action of NLR-targeting miRNAs and phasiRNAs

Despite the occurrence of miRNA-induced phasiNLR production in many plant species, only a few examples of their mode of action have been described in detail. High-throughput sequencing-based parallel analysis of RNA ends (PARE, Box 1) in Medicago truncatula provided the first evidence of phasiNLR activity, as clear signatures of their activity by generation of cleaved fragments of both primary targeted NLR transcripts (cis silencing) and secondary NLR targets (trans silencing) were found (see Zhai et al., 2011). Using RLM-RACE (Box 1), slicing activity guided by a miR482-triggered phasiRNA derived from the NLR-encoding gene was also demonstrated in Paeonia lactiflora (RPS2-like). Nonetheless, this case is of relevance since it is one of the very few examples in which changes in target endogenous levels of a secondary target (BraCP24 mRNA, at least when the two are transiently co-expressed in Nicotiana benthamiana leaves (Cui et al., 2020). This example is somewhat unusual because BraCP24 is not an NLR-encoding gene and is involved in development, not defense, and because phasiRNA130-4 arises from the miRNA of a TIR-only protein. Nonetheless, this case is of relevance since it is one of the very few examples in which changes in the trigger miRNA levels have been shown to significantly impact endogenous levels of a secondary target (BraCP24) mRNA (Cui et al., 2020).

In Arabidopsis, miR472-RDR6-dependent siRNAs have been proposed to regulate NLR abundance and the immune response against P. syringae (Boccara et al., 2014). However, experimental evidence supporting this notion is based on the analysis of the nfr6-15 null mutant, which also exhibits altered auxin responses (Allen et al., 2005; Williams et al., 2005; Fahlgren et al., 2006). More recently, analysis of Arabidopsis PARE libraries provided evidence of the activity of several phasiNLRs, triggered by miR825-5p on its main target, the TNL-encoding MIST1 transcript, on both primary (cis-silencing) and secondary (trans) NLR target genes (López-Márquez et al., 2021). Additionally, a Brassica napus phasiRNA (phasiRNA130-4) whose production is triggered by the miR1885–BraTIR1 miRNA interaction, mediates trans silencing of BraCP24 mRNA, at least when the two are transiently co-expressed in Nicotiana benthamiana leaves (Cui et al., 2020). This example is somewhat unusual because BraCP24 is not an NLR-encoding gene and is involved in development, not defense, and because phasiRNA130-4 arises from the miRNA of a TIR-only protein. Nonetheless, this case is of relevance since it is one of the very few examples in which changes in the trigger miRNA levels have been shown to significantly impact endogenous levels of a secondary target (BraCP24) mRNA (Cui et al., 2020). In fact, while PARE and/or RLM-RACE analysis have demonstrated phasiNLR-mediated

Table 1. Continued

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Speciea</th>
<th>miRNA size</th>
<th>Main miRNA targetsb</th>
<th>Target site domain/motif</th>
<th>Evidence of miRNA activity</th>
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<td>miR11519</td>
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<td>MA_395749g0010</td>
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</tr>
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<td>Glyma18g51960</td>
<td>Zhao et al. (2015)/Zhai et al. (2011)</td>
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<td>B. distachyon</td>
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<td>RNL</td>
<td>LRR</td>
<td>PARE</td>
<td>Bradi4g10030</td>
<td>Zhang et al. (2016)</td>
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</table>

a The species in which the activity of the miRNA was first validated and/or the phasiNLR biogenesis first detected. Note that the miRNA may be present in other species.

b The main subclade of NLRs targeted by a given miRNA.

c The methodology used to detect the miRNA activity over the target NLR (see Box 1).
cleavage of both cis and trans NLR target genes (Zhai et al., 2011; López-Márquez et al., 2021), limited miRNA-seq evidence supports the impact of changes in miRNA/phasiNLR levels on secondary target mRNA levels. For example, changes in miR482/2118 levels (Box 2) in tomato significantly impact disease resistance, but this clear physiological effect is hardly explained by the rather small changes detected in NLR mRNA accumulation by RNA-Seq (Canto-Pastor et al., 2019). The authors proposed that this discordance between the impact on target mRNA accumulation and on defense activation supports the notion of a mixed mode of action for miRNAs and phasiNLRs, with part of the silencing activity being carried out through translational repression. In N. benthamiana, translational repression of NLR mRNAs has been experimentally supported by transient expression of Triiteaceae-specific miR9863a and tomato miR482f followed by miRNA and protein analysis (Liu et al., 2014; Ouyang et al., 2014). siRNAs have been shown to regulate mRNA targets through translational repression in other targets (Brodersen et al., 2008; Wu et al., 2020), a phenomenon that, if demonstrated for the regulation of NLRs, would add to the complexity of this regulation and could potentially constitute an important layer of phasiNLR-dependent modulation. Alternatively, considering the typically large number of primary and secondary target NLRs under miRNA regulation, the cumulative effect of small decreases in the mRNA levels of each individual NLR, because of miRNA- and/or phasiNLR-mediated degradation might eventually amount to a significant impact on immunity, in a context that may be hard to detect by RNA-seq experiments (López-Márquez et al., 2021).

The mode of action of small RNAs is governed by its AGO partner (Martin-Merchan et al., 2023). Analyses of AGO-bound small RNA fractions have shown that different members of the AGO family may be involved in NLR regulation, with a substantial amount of TNL-derived phasiRNAs bound to AGO1 and AGO2 in Arabidopsis (López-Márquez et al., 2021). Clearly, differences in AGO activity domains, their subcellular localizations, molecular functions, or even their expression profiles under stress conditions (e.g. pathogen attack) could influence the activity of phasiNLRs. It is noteworthy that the number of encoded AGO proteins is highly variable in plants (Martin-Merchan et al., 2023), a phenomenon that may contribute to functional specialization. Thus, future research in other plant species encoding a larger number of AGO proteins and phasiNLR loci (e.g. soybean) may further elucidate how AGO–phasiNLR networks operate.

Movement of siRNAs affects silencing effectiveness and has broad implications for plant development and immunity (Chitwood et al., 2009; Shine et al., 2022). While many miRNAs tend to act within a short distance of their biogenesis site (10–15 cells), siRNAs generated through RDR6-dependent amplification are more prone to move between cells, contributing to spread of the silencing signal (Felippe
et al., 2011). Therefore, it is tempting to speculate that phasiNLRs may act to expand the domain of NLR repression beyond the expression domain of the trigger miRNA. However, this aspect of phasiNLR function remains to be experimentally explored.

miRNA/phasiNLR regulation in response to pathogen detection

Intracellular NLR receptors have been considered for a long time as canonical elements of ETI, whereas membrane-anchored pattern recognition receptors (PRRs), activated upon perception of PAMPs such as bacterial flagellin, are canonical elements of PTI (Jones and Dangl, 2006). However, there is increasing evidence of considerable crosstalk and synergy between PRR-dependent (PTI) and NLR-dependent defense responses (ETI) (Fig. 3) (Hatsugai et al., 2017; Kadota et al., 2018; Lapin et al., 2020; Pruitt et al., 2021 Ngou et al., 2021; Yuan et al., 2021b). Activation of PRR and NLRs is triggered by ligands in different subcellular locations, but signals initiated from PRR and NLR converge upon common signaling elements (Lu and Tsuda, 2021). Additionally, a full NLR-dependent (ETI) response in Arabidopsis requires PRR-dependent activation (PTI) of some downstream components, such as membrane-bound NADPH oxidases or MAP kinases (Dongus and Parker, 2021; Ngou et al. 2021; Yuan et al., 2021b). Furthermore, a subset of PRRs establishes PTI defense responses with the contribution of signaling components traditionally associated with NLR-activated signaling, such as the EDS1–PAD4 module (Pruitt et al., 2021; Tian et al., 2021; Feehan et al., 2023). This interplay between PRR- and NLR-mediated immune signaling has many implications for the analysis and characterization of plant–pathogen interactions and for our understanding of the role of miRNA/phasiNLR post-transcriptional regulation of NLRs in the plant responses against different biotic stresses. One mechanism by which activation of PRR signaling has been proposed to potentiate NLR-mediated defenses is through the regulation of gene expression (Ngou et al., 2022). Transcriptional activation of multiple NLR genes has been shown to take place following PRR activation (Bjornson and Zipfel, 2021; Tian et al., 2021). Although the mechanisms behind this link are not yet fully established, mitogen-activated protein kinase (MAPK) cascades and transcription factors (TFs) are likely to be involved (Ngou et al., 2022). Alleviation of miRNA/phasiTNL silencing of NLR genes in response to PRR activation would contribute to establish such a connection.

Several reports have shown that miRNA-NLR/phasiNLR levels respond to pathogen sensing. The levels of the mature 22 nt miR472 are reduced after treatments with flg22 (poplar and Arabidopsis) or fungal PAMPs (Arabidopsis) (Li et al., 2010; Boccara et al., 2014; Su et al., 2018; Vasseur et al., 2022, Preprint). Treatments with flg22 also lead to reduced levels of both the precursor and the mature miR825-5p in Arabidopsis (López-Márquez et al., 2021). Thus, PRR signaling alone is sufficient to trigger changes in the levels of precursors and/or mature levels of miRNA in different plant species. The mechanism linking PRR activation to down-regulation of the levels of precursor and mature miRNAs involves transcriptional repression (Su et al., 2018; López-Márquez et al., 2021), but remains largely unexplored. Repression may also take place at the level of mature miRNA and phasiNLR production, since RDR6 and AGO1 mRNA levels have been shown to rapidly decrease in Arabidopsis leaves and seedlings upon flg22 treatment (Boccara et al., 2014). Whether NLR signaling alone could also trigger changes in the levels of precursors and/or mature levels of miRNA/phasiNLRs has not been investigated. Investigation of such a scenario would require analyses of miRNA/phasiNLR levels in transgenic plants expressing ETI-triggering effectors, since the use of bacterial delivery systems or Agrobacterium-mediated transient expression will produce confounding effects due to PRR activation. Thus, a model arises where NLR levels are kept low in the absence of the pathogen partly owing to miRNA/phasiNLR-mediated gene silencing, and these levels increase in response to pathogen-induced PRR-mediated signaling through both transcriptional activation of NLR-coding genes (Bjornson and Zipfel, 2021; Tian et al., 2021) and alleviation of miRNA/phasiNLR repression (Fig. 3). In this model, miRNA/phasiNLR regulation would constitute an additional link between PRR- and NLR-mediated responses, with PRR-mediated pathogen perception increasing NLR protein levels.

Role of miRNA/phasiNLR regulation in immunity

It is now clear that pathogen detection affects the levels of trigger miRNAs, but how does miRNA/phasiNLR regulation impact the outcome of the pathogen–host interaction? Most reports characterizing the impact of miRNA/phasiNLR regulation on plant immunity have shown that changes in the levels of these miRNAs (Box 2) alter resistance against virulent pathogens (i.e. that either successfully suppress ETI or trigger weak or no ETI) (Li et al., 2012b; Boccara et al., 2014; Canto-Pastor et al., 2019; López-Márquez et al., 2021; Vasseur et al., 2022, Preprint). In this context, alleviation of miRNA/phasiNLR repression leading to NLR increases could contribute to increase resistance by enhancing either PRR-activated responses or the level of ETI. Changes in the levels of some of these miRNAs (e.g. miR825-5p or miR472) specifically alter elicitation of plant immune responses by flg22, such as reactive oxygen species (ROS) production, callose deposition, or MAPK activation (Boccara et al., 2014; Su et al., 2018; López-Márquez et al., 2021). Thus, the NLRs regulated by these miRNA/phasiNLR modules are likely to be relevant for PRR-mediated immunity in the absence of specific NLR.
Small RNA and the regulation of NLR-mediated plant immunity

Tian et al. (2021) showed that up-regulation of multiple TNLs boosts the immune responses against a *P. syringae* DC3000 *hrcC* mutant (which fails to introduce effectors into the host cell and therefore does not induce ETI) in Arabidopsis. The authors proposed that increases in the dosage of so many TIR genes could lead to an increased production of small molecules with defense signaling activities even in the absence of effector detection, assuming a certain degree of NLR misfiring, thus activating downstream TIR signaling pathways to a level sufficient to reinforce PTI responses without triggering cell death (Fig. 3). Nonetheless, given the increasing number of molecular links established between PRR and NLR immune signaling, several options are open to explain how increased levels of multiple NLR genes, many uncharacterized, can contribute to reinforce PRR-mediated immunity. For instance, a subset of the many uncharacterized miRNA/phasiNLR-regulated NLRs might act as helpers activated by EDS1–PAD4 complexes involved in PRR-mediated signaling, thus influencing the PTI response.

In Arabidopsis, the NLR-coding *RPS5* gene is a target for miR472-mediated silencing (Boccara et al., 2014). *RPS5* mediates ETI and HR activation in response to the bacterial

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**Fig. 3.** Overview of miRNA/phasiNLR regulation in relation to PRR- and NLR-mediated immunity. Schematic view of PRR- and NLR-mediated defense signaling and their connections. Solid arrows represent known defense signaling events. Dashed arrows show signaling processes not described at the molecular level. PRRs are modeled after the FLS2 receptor of bacterial flagellin and its co-receptor BAK1. The main components contributing to extracellular ROS signaling (RBOH and calcium channels) are depicted. RLCK, CPK, and MAPK cascades are represented generically. Effector activation of NLRs is shown with solid arrows while effector activities related to NLR-mediated PTI enhancement or silencing are indicated as dotted arrows. The figure was created with BioRender.com.
effectors AvrPphB (also known as HopAR1) (Simonich and Innes, 1995; Ade et al. 2007). Growth of a P. syringae DC3000 derivative expressing AvrPphB, which is usually limited by AvrPphB-triggered RPS5-dependent ETI, was significantly increased in plants overexpressing miR472, in keeping with a significant decrease of RPS5 transcript levels. Since AvrPphB was delivered from a bacterial pathogen in this experimental setting, PRR activation may have occurred concomitantly with RPS5 activation. Such PRR activation is, however, likely to be residual because of its suppression by pathogen effectors. Interestingly, the authors did not detect any significant changes in bacterial growth of P. syringae DC3000 expressing the effector AvrRpt2, whose activity is directed by CNL RPS2 that is not a target of miR472. This observation supports the notion that the miR472 effect on signaling mediated by RPS5 activation was specific (Boccara et al., 2014). In another study, mRNA accumulation of the defense marker gene PR1 in response to a B. syringae DC3000 derivative expressing AvrRpt2 was significantly increased in MIR825-silenced Arabidopsis plants. RPS2 is not a known target for miR825/phasiNLR-mediated silencing (López-Márquez et al., 2021). In this case, RPS2 may have been induced indirectly as a consequence of the alleviation of miR825-5p suppression on primary and secondary targets. Additionally, in N. benthamiana, Agrobacterium-mediated transient expression of miRNAs miR6019 and miR6020, which target transcripts of the NLR-coding gene N, attenuates N-mediated resistance to tomato mosaic virus (TMV) (Li et al., 2012b).

Both miR472 and miR825-5p have been linked to the activation of the induced systemic response (ISR) in Arabidopsis (Niu et al., 2016; Nie et al., 2019; Jiang et al., 2020). The ISR is triggered by beneficial microbes and results in the activation of defense responses in local and systemic tissues that lead to a primed state where the plant can mount a faster and stronger cellular response upon pathogen attack (Conrath et al., 2002). Levels of miR472 and miR825-5p decrease when ISR is activated (i.e. pre-treatment with Bacillus cereus) prior to infection with either P. syringae or B. cinerea, although this decrease is only statistically significant if pathogen infection follows ISR activation (i.e. treatment with B. cereus followed by P. syringae or B. cinerea infection) (Niu et al., 2016; Nie et al., 2019; Jiang et al., 2020). Whether changes in the levels of these miRNAs impact the ability of Arabidopsis to establish an ISR remains to be established.

Why is miRNA/phasiRNA regulation of NLRs so prevalent?

NLR regulation by miRNAs, often involving 22 nt miRNAs and phasiNLR production, is widespread among different plant lineages (Table 1; Zhang et al., 2016). This recurrent pattern supports the importance of both regulatory nodes and begs the question of why NLR regulation through this combination of small RNA activities is so important. In the closing paragraphs, we discuss different possible explanations, none of them mutually exclusive, for this interesting question.

Leveraging growth/defense dichotomy

Appropriate homeostasis of NLR activity is critical for plant performance (Li et al., 2015). This condition is typically achieved by low-level expression under favorable growth conditions, and up-regulation in response to pathogen perception (Xiao et al., 2003; Zipfel et al., 2004). Indeed, effective activation of NLR-mediated defense signaling requires NLR protein levels to be above a certain threshold (Bieri et al., 2004; Holt et al., 2005). However, exacerbated NLR levels can cause autoimmune defects that include plant growth retardation, yield penalties, and, sometimes, necrosis (Mackey et al., 2003; Xiao et al., 2003). A simple, perhaps too simple, explanation for the importance of miRNA/phasiNLR down-regulation of NLR protein production in the absence of a pathogen is to achieve finely tuned NLR expression to reduce potential NLR-associated fitness losses (Li et al., 2012b; Shivaprasad et al., 2012; Fei et al., 2013; Boccara et al., 2014). This idea has been investigated by measuring the impact on plant fitness of elimination or exacerbation of miRNA/phasiNLR regulation. In Arabidopsis, overexpression of miR472 does cause a growth advantage in unchallenged conditions, although this advantage is small and without major effects on plant development. Conversely, knockdown and knockout of miR472 cause small growth delays (Jiang et al., 2020). An independent and more detailed study confirmed these results and showed that reduced levels of miR472 affected rosette growth, but not the age at bolting (Vasseur et al., 2022, Preprint). Also, in Arabidopsis, knockdown of both miR825-5p and miR825-3p, the two functional miRNAs generated from the same precursor, reduced plant growth, although in this case overexpression of both using artificial miRNAs did not have any impact (Niu et al., 2016). It is possible that stronger fitness costs could be associated with the loss of miRNA/phasiNLR regulation in other species where it is more prevalent (Zhang et al., 2016). However, knockdown of miR482 in tomato was not associated with gross effects on growth or development (Canto-Pastor et al., 2019). In conclusion, the available evidence suggests that the miRNA/phasiNLR system is relevant for plant fitness, although the effects of its manipulation are relatively minor in unchallenged laboratory settings. In this model, the combined 22 nt trigger miRNA/phasiNLR activity could perhaps be associated with a larger reduction of fitness costs than simple repression by 21 nt miRNAs. In any case, these studies have analyzed the potential impact on fitness of the loss of miRNA/phasiNLR regulation in optimal growth conditions. Stronger impacts on plant growth are expected to be associated with lower levels of these miRNAs when growing in less favorable conditions, since limitations on nutritional availability, or abiotic stresses such as drought, extreme temperatures, or...
humidity seem to exacerbate any trade-offs between growth and resistance (Ariga et al., 2017; Karasov et al., 2017).

Considerations on the kinetics, amplitude, and duration of NLR induction

While increasing NLR protein levels via PRR signaling after pathogen detection (Navarro et al., 2004; Björnson and Zipfel, 2021; Tian et al., 2021) is obviously relevant, the kinetics of its induction, control of its amplitude, and its timely repression might be as important. In this regard, the miRNA/phasiNLR regulatory module responsive to PRR-mediated pathogen detection (Shivaprasad et al., 2012; Bocca et al., 2014; Su et al., 2018; López-Márquez et al., 2021; Vasseur et al., 2022, Preprint) may be particularly beneficial.

With regards to induction kinetics, it bypasses the need for mRNA synthesis and processing from NLR-encoding genes, and is, therefore, a fast and efficient way to boost NLR production and hence the immune system at large following pathogen attack. The increase in NLR expression licensed by the reduction in the levels of trigger miRNAs has indeed been shown to enhance NLR-dependent quantitative resistance through potentiating PRR-mediated responses in the absence of specific effector-mediated activation of any NLR (Bocca et al., 2014; Su et al., 2018; López-Márquez et al., 2021; Ngou et al., 2021; Yuan et al., 2021a). It has also been proposed that the advantage of combined 22 nt miRNA/phasiRNA over direct 21 nt miRNA action in the regulation of NLRs is the possibility to expand the network of NLRs regulated by a single trigger miRNA (Zhai et al., 2011; Shivaprasad et al., 2012; Bocca et al., 2014; López-Márquez et al., 2021), thereby further enhancing the response amplitude and broadening the range of pathogens targeted.

Because of the potency of NLR signaling to restrict growth and induce cell death, uncontrolled response amplitudes and durations are not desirable. The miRNA/phasiNLR regulatory module may also be beneficial. For example, after pathogen perception, the increased availability of target NLR miRNAs triggered by PRR activation may sustain phasiNLR production even when trigger miRNA levels decrease (López-Márquez et al., 2021). Such a fast, but gradual, increase in NLR mRNA levels may contribute to a controlled deployment of defenses and also may contribute to explain the subtle impact of experimentally altered trigger miRNA levels on defenses, including PTI (Li et al., 2012b; Bocca et al., 2014; López-Márquez et al., 2021; Vasseur et al., 2022, Preprint), RPM5-mediated ETI (Bocca et al., 2014), and ISR (Niu et al., 2016; Nie et al., 2019; Jiang et al., 2020). In such a scenario, miRNA/phasiNLR regulation would not cease immediately upon pathogen attack and could be important to fine-tune NLR levels throughout the plant response against the pathogen. It can also be hypothesized that miRNAs and phasiRNAs may help to quickly bring back levels of NLRs, whether involved in boosting PRR-mediated or effector-triggered NLR-activated immunity, to their steady-state levels once the incoming threat has been controlled, thus resetting the system. Such a reset of mRNA target levels has been previously reported (Liu et al., 2014; López-Márquez et al., 2021). Thus, the combined miRNA/phasiNLR system may help avoid potential side effects of a disproportionate immune activation by tuning both the amplitude and the duration of the response correctly. An additional physiological consequence of such control could be to act as a barrier against excessive NLR-dependent cell death, a process exploited by necrotrophic pathogens (Lorang et al., 2012; Pitsili et al., 2020).

Benefits of employing a non-cell-autonomous silencing system

The involvement of phasiNLR in NLR regulation during defense deployment opens up the interesting possibility that differences in movement between miRNAs and siRNAs may imply the combination of cell-autonomous and non-cell-autonomous control of NLR production (Felippe et al., 2011). If phasiNLRs share this ability to engage in pronounced non-cell-autonomous action with other amplified siRNAs, it could be important in preventing run-away cell death, helping to keep in check the hypersensitive response trigger upon NLR-activated ETI. In this aspect, phasiRNA could also provide additional benefits by controlling the spatial reach and amplifying the reset silencing process.

Trigger miRNA/phasiNLR control as a means of RNAi-directed pathogen surveillance

Many pathogens with completely different life styles including viruses, bacteria, oomycetes, and fungi employ effectors capable of suppressing RNA silencing to manipulate host gene expression programs and/or suppress the RNA silencing involved in defense (Anandalakshmi et al., 1998; Béclain et al., 1998; Kasschau and Carrington, 1998; Voinnet et al., 1999; Navarro et al., 2008; Qiao et al., 2013; Csorba et al., 2015; Hou et al., 2019). Therefore, the miRNA/phasiNLR-based regulation of NLRs may constitute a simple mechanism to sense such anti-RNAi effector activities to release NLR expression to switch on the immune response (Li et al., 2012b; Shivaprasad et al., 2012; Fei et al., 2013, 2016). Importantly, pathogens do not just target the miRNA branch of RNAi pathways; some target DICER-LIKE proteins involved in siRNA generation and others target proteins such as SGS3 or RDR6, key components of amplified RNA silencing (Csorba et al., 2015). The trigger miRNA/phasiNLR system ensures that many NLR-encoding genes are under the control of at least one branch of RNAi acting at the post-transcriptional level (Axtell, 2013; Bologna and Voinnet, 2014; Borges and Martienssen, 2015). This setting may therefore allow plants to sense any pathogen that uses an effector to target any step in post-transcriptional RNAi,
as suggested by Vasseur and collaborators (Vasseur et al., 2022, Preprint). Seen in this way, the trigger miRNA/phasiNLR system may be viewed as a variant guard model for indirect sensing of the broadest possible spectrum of RNAi-targeting pathogen effectors. Instead of confonrnational changes of a single NLR leading to its activation, this surveillance system would react by increasing NLR expression, thereby probably increasing the likelihood that some NLRs wind up in the active conformation to induce immune responses.

Open questions for future research

Despite progress, the precise roles, and modes of action of phasiRNAs targeting NLRs are still ill defined. Several relevant aspects of their mode of action will require further research. (i) Are phasiNLR more prone to operate through translational repression or by cleaving the target mRNA? (ii) Can phasiNLRs move in a non-cell-autonomous manner reaching miRNA-free tissues as has been reported for other siRNAs? And if so, is this a relevant aspect on immune regulation? Moreover, there are additional open questions on the field that are relevant for understanding how this mechanism of regulation operates. A deeper search into siRNA libraries (http://ipf.sustech.edu.cn/pub/asrd; Yu et al., 2017; Feng et al., 2020) unraveled that in Arabidopsis both miR472 and miR825-5p are associated with AGO10 (ZWILLE), the closest paralog of AGO1 that was proposed as a transitivity inducer (Iki et al., 2018). However, AGO10 mediates the translational repression of miRNA targets (Brodersen et al., 2008; Mallory et al., 2009; Martin-Merchan et al., 2023) and competes with AGO1 for miR165/166 binding, inhibiting the function of those miRNAs during apical shoot development and triggering their degradation (Zhu et al., 2011; Yu et al., 2017). AGO10 also sequesters miR398 and prevents its movement (Cai et al., 2021), a result that supports the role of AGO10 as a miRNA locker (Manavella et al., 2011). These observations open up relevant questions such as the following. (i) Are these two proteins contributing to phasiNLR biogenesis through miR472 and miR825-5p? (ii) Is the partitioning of those miRNAs onto AGO1 and AGO10 relevant for their mode of action (slicing versus translational repression)? Or may it vary according to AGO1/AGO10 expression patterns? And finally, (iii) is AGO10 acting as a miRNA locker impeding NLR regulation in some specific tissues?

It is also intriguing how the large differences in the number of miRNAs involved in NLR regulation and that of phasiNLR loci existing between different species may affect the mode of action or the possible signaling networks involved in regulating NLR function in these species. Future analysis comparing the NLR networks and functional impacts on such different systems could provide insightful information on both the mechanistic implications and how these differences evolved.

In this regard, generation of a few miRNA loss-of-function mutants (knockouts) in selected species via the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR–associated protein) technology would provide valuable information.

Another aspect important for understanding the relevance of this type of regulation in plant defenses that has not been investigated at the molecular level is how NLR-targeting miRNAs and phasiNLRs respond to PRR-mediated pathogen perception. The link between PAMP perception and decreases in transcription of the MIR loci and mature miRNA levels is clear; however, the signaling elements involved are still unknown. Additionally, mature miRNA levels have been shown to drop as soon as 30 min after PAMP treatment (Boccara et al., 2014; Su et al., 2018; Vasseur et al., 2022, Preprint). However, the longer average half-lives of miRNAs in other systems (Marzi et al., 2016; Reichholf et al., 2019) suggest that plant cells may possess a mechanism to quickly get rid of those miRNAs and boost the immune response. It is tempting to speculate that the appearance (in terms of evolution) of endogenous target mimics or sponges may be an easy explanation for these observations, resembling the case of miR399 and the endogenous target mimic IPS1 during phosphate starvation (Franco-Zorrilla et al., 2007). It is important to note that plant genomes contain a number of non-coding NLR-derived transcripts of uncharacterized function. These transcripts may serve as a template for phasiNLR biogenesis, as is the case for TAS5 in tomato (Li et al., 2012a; Canto-Pastor et al., 2019). However, due to sequence similarities, these long non-coding RNAs may constitute the source RNA molecules from which miRNA/phasiNLR sponges could originate.

Solanaceae-specific 22 nt miR6019 triggers the production of phasiNLR from the target NLR N gene conferring resistance against TMV in Nicotiana tabacum (Li et al., 2012b). However, miR6019 has been shown to be transcriptionally repressed during plant maturation, a phenomenon accompanied by heightened levels of N mRNAs and increased N-mediated resistance against TMV in tobacco plants, a mechanism that may be conserved in other Solanaceae species (Deng et al., 2018). Thus, this work could be considered the first to establish adjustments of a ‘miRNA–phasiNLR module’ linked to plant development that could be important to fine-tune NLR expression during this process. Additional work on this potential link in other species and regulatory modules may be important to understand the relevance of this type of regulation and to explain its prevalence.

Much have been discovered about the role of 22 nt miRNA and phasiNLR regulation in plant immunity in the last decade. This, in conjunction with the developments in the last few years in defense signaling, make this a hot and exciting topic with a great potential to be unlocked in generating new strategies for crop protection, in which much is yet to be learnt.
Conflict of interest

No conflict of interest declared.

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